INTER-INDIVIDUAL VARIATION IN GENE EXPRESSION IN TORPID AND INTERBOUT EUTHERMIC ARCTIC GROUND SQUIRRELS

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INTER-INDIVIDUAL VARIATION IN GENE EXPRESSION IN TORPID AND INTERBOUT EUTHERMIC ARCTIC GROUND SQUIRRELS

A

THESIS

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By

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Abstract

Alaskan Arctic ground squirrels, *Spermophilus paryii*, hibernate about seven months per year. During two-week torpor periods, respiration, circulation, metabolism, and catabolism are dramatically decreased, except for brief periods of interbout euthermia. These divergent hibernation states provide a particularly compelling model for variance-based studies of global gene expression. A guiding hypothesis in this Thesis is that Arctic ground squirrels exit interbout euthermia and enter torpor with an invariant metabolic scaffolding of various metabolites that are erected to serve as a ready metabalome for the challenges of the next brief return to euthermia. To develop this hypothesis further, I performed an exploratory data analysis of high-density mouse cDNA microarrays cross-hybridized with Arctic ground squirrel mRNA to measure transcriptomes in brown adipose, skeletal muscle, and liver tissues. The results revealed that variation in transcript expression profiles were tissue specific and may reflect the degree to which tissues are active during hibernation. These results are encouraging. They justify a more thorough evaluation of the utility of using global variation in transcript expression patterns. In combination with *a priori* biological knowledge, these patterns will guide future studies into more detailed analyses of hibernation-state dependent and functionally relevant transcripts.

Dedication

This Thesis is dedicated to my parents, Harold and Sally Burman; my partner, Peggy Kompalla, my advisor, Abel Bult-Ito, the other committee members, Jeff Shrager and Roy Bird, Larry Duffy and Sheila Chapin from the Chemistry Department, and the entire Wistar Group.

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Acknowledgments

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1. Introduction

Arctic ground squirrels, *Spermophilus paryii*, range widely in much of Alaska, especially in the interior and northern regions. The hibernation season for these animals runs roughly from late August/mid-September until the beginning of the Arctic summer (Buck and Barnes, 2000). This period of torpor is characterized by dramatically reduced levels of respiration, circulation, metabolism (e.g., transcription and translation), and catabolism (e.g., proteolysis) (van Breukelen and Carey, 2002), except for brief periods of interbout euthermia. Body temperature during torpor is maintained near -2°C (Buck and Barnes, 2000). Interbout euthermic periods occur with a markedly regular periodicity of about twice monthly when a hibernating Arctic ground squirrel will raise its respiration, blood flow and body temperature to euthermic levels for roughly two days (Boyer and Barnes, 1999; Buck and Barnes, 2000). The timing of these interbout euthermic episodes is extremely well maintained. The precise periodicity is, however, highly dependent on the individual Arctic ground squirrel (Boyer and Barnes, 1999). During the interbout euthermic period, neither ingestion nor evacuation occur (Humphries, et al., 2001; Lesser, et al., 1970). Periodic arousal during the hibernating season not only requires a great expenditure of energy but leaves the Arctic ground squirrel vulnerable to many physiological stressors (not least of which is the sudden influx of oxygen-rich blood) (Chauhan, et al., 2002; Storey, 2004). The benefits of these physiologically and energetically taxing interbout euthermic episodes remain unclear (Lovegrove, et al., 1999), although a great deal of work continues to be devoted not only to addressing these questions but to elucidating the mechanisms involved.

Many of the most commonly employed approaches to high-throughput gene expression analysis are predicated on assessing differential levels of expression of transcripts under different types of experiments. Examples include comparisons

between samples of different physiological states or conditions (e.g., healthy subjects vs. those afflicted with a particular pathology) and time course measurements. The emphasis on fold-change in transcript expression levels is an understandable, yet unfortunate, trend whose primacy has extended to the current and welcome emergent technologies allowing for high-throughput RNA analysis. Nucleic acids are still by far the most amenable biomolecules for high-throughput study in terms of necessary labor, cost and current technology available to most of today's bioinformaticists. These comprise just one of many families of metabolites which may contribute to a minimal metabolic scaffolding or any other cellular state. Much of the physiologically relevant activity in the cell may not be reflected in the transcriptome; certainly not universally mappable to fold changes in expression levels. Profound and abrupt changes occur in the cell without any transcriptional input. This is often seen in allosteric enzymes switching their function by being altered by a ligand or through alteration by differential phosphorylation, oxidative state, response to extracellular stimuli, etc.

Another concern with the reliance on transcript fold change to provide most of the information that a gene expression profile can provide is that it potentially overlooks the importance of genes that might stay transcriptionally uneventful, yet serve a variety of functions in different physiological milieus. An example of such a transcript would be aco1, which is normally known as an RNA template for the enzyme Aconitase, a crucial constituent of the citric acid cycle. In the presence of reactive nitrogen species, often induced by iron oxidation, the same RNA molecule adopts a new conformation as the Iron Response Element halts translation of the expressed gene for Ferratin, which is involved in iron homeostasis (Haile, et al., 1992; Moeder, et al., 2006; Theil, 1993; Zheng, et al., 1992).

This is not to say that no studies in transcription profiles have been performed that are sufficient in explaining the underlying biological changes in question. Many of the studies that focus solely on fold change in RNA expression levels have often led to valuable discoveries both in classical gene by gene studies and in modern high-throughput differential expression studies involving up to hundreds of thousands of genes in simultaneity (see for example, Kim, et al., 2005; Klemke, et al., 2006; Mendis, et al., 2005; Sclabas, et al., 2005; Shukla, et al., 2005; Vleugel, et al., 2005).

This study, however, posits a reassessment of the primacy of utility of examining fold change in transcript levels to a more careful scrutiny of the variances of transcript expression levels. The highly complex dynamic systems of most biological processes often necessitate tightly coordinated levels of numerous metabolites and, by extension, those of numerous RNA transcripts.

The interbout euthermic periods of hibernation provide a particularly compelling model for variance-based studies of global gene expression. A guiding hypothesis in this Thesis is that an Arctic ground squirrel exits interbout euthermia and enters torpor with an invariant metabolic scaffolding of proteins, transcripts and any number of other metabolites that are erected not so much to address immediate physiological challenges; rather, to serve as a ready metabalome for the challenges of the next brief return to euthermia. As no transcription or translation occurs during torpor (Haile, et al., 1992; Knight, et al., 2000; Moeder, et al., 2006; Storey, 2003; Storey and Storey, 2004; Theil, 1993; van Breukelen and Martin, 2001; Zheng, et al., 1992), any transcripts found in a torpid Arctic ground squirrel will have been transcribed before exiting the interbout euthermic period. Because this is about two weeks before entry into the next interbout euthermic period, when a number of immediate challenges confront the Arctic ground squirrel, a subset of genes is hypothesized to be anticipatory and, thus, invariant in a torpid Arctic ground

squirrel. A set of early response transcripts will have to be available and ready to be translated, such as genes involved in cell-cycle arrest, metabolism, oxidative stress pathways, and homeostasis. This would be analogous to a fertilized egg containing maternal transcripts for the regulation of the initial developmental stages. As comparisons among states are a large part of this Thesis, the point needs to be made that during hibernation, torpor is the condition that can best be described as a distinct state. The interbout euthermic hibernation state is more variable, i.e., may comprise several sub-states.

High-density mouse cDNA microarrays cross-hybridized with Arctic ground squirrel mRNA were used primarily to examine the existence of such an invariant metabalome and to elucidate whether metabolomes are expressed differently in three different tissue types. Because of the limited sample size as a result of using a limited wild resource, i.e., Arctic ground squirrel individuals, this Thesis will not be equipped to proffer conclusive statements with the strength of robust statistics. Instead, the intent and expectation of this study is to be exploratory and examine the veracity of such a hypothesis and help bring light to experiments and experimental designs which have the most promise of fruition in future studies.

2. Materials and Methods

2.1. Sample Collection and Preparation

The methods employed in this study were described in detail in Yan, et al. (2006). In brief, RNA from the Arctic ground squirrels was isolated in Alaska using the method of Chomzynski and was poly(A+) enriched using an oligo-dT cellulose matrix. Microarrays were ^{33}P labeled and hybridized to MA07 embryonic mouse cDNA arrays. Hybridization was carried out for 16 hours at 42°C, followed by 24 hours at 35°C in 5 ml MicroHyb buffer (Research Genetics). The PolyA RNA was then T7 linearly amplified at the Wistar Group. Filters from the hybridizations were batch washed in a large container for consistency. Filters were rinsed at room temperature with 2 x SSC buffer in a temperature-controlled shaking water bath. Filters were washed twice for 30 min in 2 x SSC buffer at 50°C, then once for 30 min in 0.5 x SSC buffer, and subsequently exposed to phosphoimager screens for 6 days and scanned at 50 M resolution in a Storm Phosphoimager. After the first scanning, the filters were rewashed in 0.1 x SSC buffer, exposed to the Phosphoimager screens for 10 days, and rescanned for the second time. Image analysis was performed with the ImaGene program. All steps subsequent to the Poly(A) enrichment were carried out at the Wistar Group at the University of Pennsylvania in Philadelphia.

2.2. Data Preparation

Spots where $\frac{Sig_{med} - BG_{med}}{BG_{med}}$ or $\frac{Sig_{med}}{BG_{med}} - 1 < 2$, where Sig_{med} is the signal median and BG_{med} is the background median, as assessed by an ImaGene image reader, were deemed to have too high a signal to noise ratio for inclusion. Furthermore, in all comparative investigations, any spot excluded from any array was excluded from all arrays being compared. Normalization was carried out in three stages:

- The signal intensities for each spot were $\log_2$ transformed.

- For each $\text{array}_i = \mathbf{A_i}$ spots_j was array-wise normalized by $\frac{\log_2 (S_j)}{\sum_{s \in \mathbf{A}_i} \log_2 (s)}$

- Spots for each array were then unit-vector scaled. Because the medians and means of all arrayed genes were on widely varying scales, the variances of these genes were unlikely to demonstrate a well-behaved probability density function and, therefore, essentially be uncomparable in the present form. Assuming that the expression of each individual gene was distributed normally, albeit within disparate ranges, their variances were non-dimensionalized and made comparable by multiplying each datum by the standard deviation / mean for each spot.

2.3. Data Normalization

Unit-normalized values between the two individuals harvested during both torpor and interbout euthermia were compared for three tissue types, including brown adipose, liver, and skeletal muscle tissues. In general, the normalized data for the gene expression patterns of the two individuals in each group are strongly correlated and, thus, comparable. As expected, the $\log_2$-transformation of the unit-normalized values greatly reduced the heteroscadacity that was present in the untransformed data. The distributions of untransformed signal intensities were, not unexpectedly, heavily skewed to the left as many of the measured values were very low and the strict lower limit of 0 prevents the open-ended expansion ability found at the upper end of the spectrum. Even after the 0-limit stricture was relieved by the use of logarithms, the density distributions failed to adopt normality and remained heavily left skewed (see Figure 3.4).

3. Results and Discussion

3.1. Regression Verification

The correlation of transcript expression levels between two squirrels was, with very few exceptions, greatest when measured in the same tissue type rather than when comparing that of different tissue types (data not shown). This result was expected because distinct tissues have disparate functions regulated by different sets of transcripts; these sets of transcripts are themselves regulated by the distinct physiology of the tissue.

The difference in correlation coefficients between the two torpid and two interbout euthermic squirrels was the largest for brown adipose tissue (0.915 and 0.782, respectively). Interestingly, the correlation coefficients of the BAT-IBE 2 squirrel with the two BAT-torpid animals were very similar to that of the two BAT-torpid squirrels (Figure 3.1). In contrast, the correlation coefficients of the IBE 1 squirrel with the two torpid animals were much lower and similar to that of the two IBE. This finding suggests that the variance in transcript expression levels of the IBE 2 squirrel is similar to that of the two torpid animals. The BAT-IBE 1 squirrel, however, apparently had transcript expression patterns quite different from the other three squirrels. This finding is consistent with an interpretation that the interbout euthermic hibernation state is more variable, i.e., comprises numerous substrates, while the torpid hibernation state is distinct and well defined. The BAT-IBE 2 squirrel may have been very close to reentering torpor and, therefore, may already have erected much of the hypothesized transcriptional scaffolding of the torpid state. On the other hand, the BAT-IBE 1 squirrel may have been in a state of very dynamically active transcription that would have introduced additional variation, including transcripts specific for processes occurring during the interbout euthermic period and metabolites maintaining the torpid state.

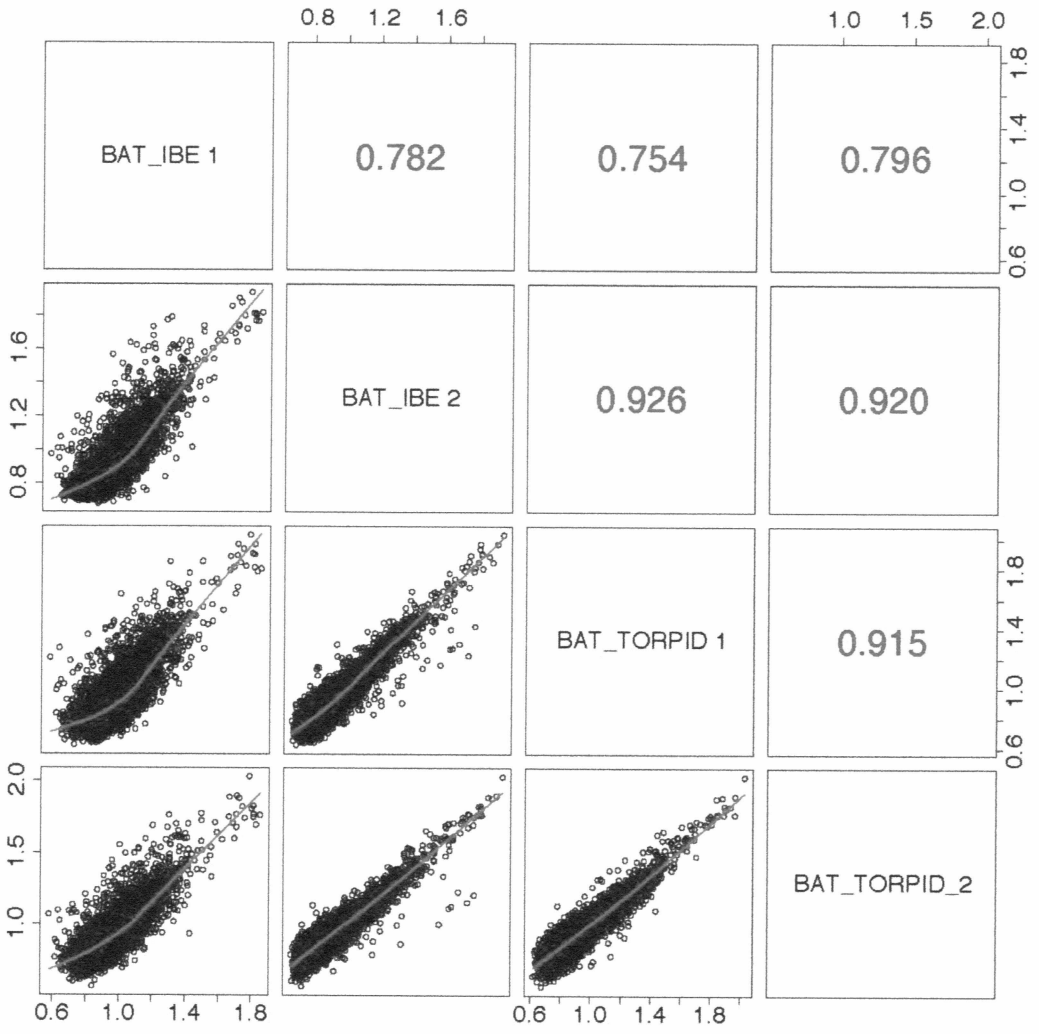

Figure 3.1. Scatter Plot Matrix of Log Normalized Transcript Abundance of Brown Adipose Tissue Among Torpid and Interbout Euthermic Arctic Ground Squirrels. Log normalized transcript abundance measures were pair wise compared among two torpid and two interbout euthermic (IBE) Arctic ground squirrels. Below the diagonal, the gene expression profiles for each pair of individuals are regressed and the traveling local mean is shown in red. The corresponding correlation coefficients for each plot are shown above the diagonal.

For liver tissue, the correlations between two squirrels were very high, with all measured correlation coefficients above 0.9 (Figure 3.2). Comparison of the two torpid transcriptomes showed a slightly higher correlation than the two interbout euthermic squirrels. The similarity between torpid and interbout euthermic expression variance profiles in all tissues suggests that the set of distinct transcripts whose difference in variation reflects the difference in states is a relatively small subset of the entire transcriptome. The IBE/torpid similarity was most pronounced in the liver tissue measurements.

For skeletal muscle tissue, the correlations between two squirrels were also very high (Figure 3.3). Interestingly, for this tissue the IBE 1 tissue was more similar to the torpid squirrels than the IBE 2 tissue, which is opposite to what was observed for brown adipose tissue.

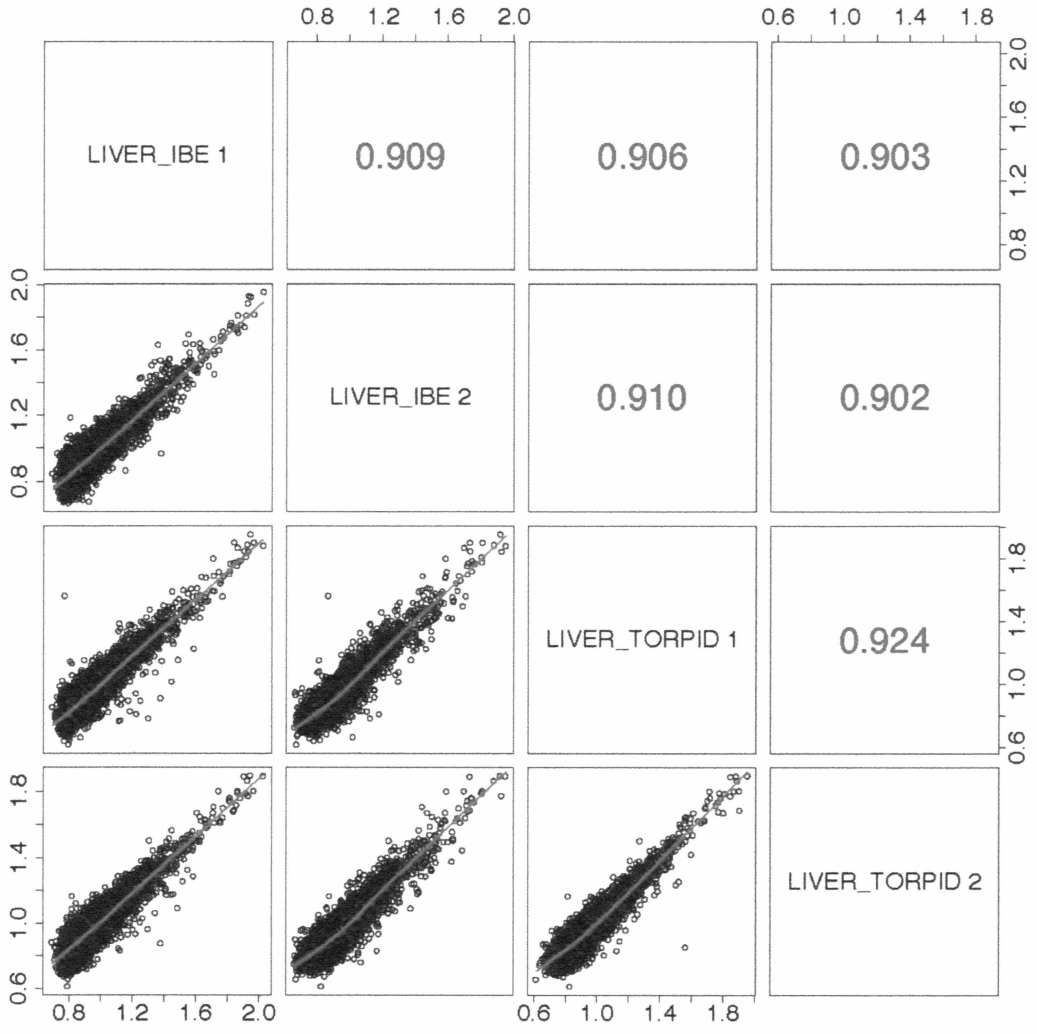

Figure 3.2. Scatter Plot Matrix of Log Normalized Transcript Abundance of Liver Tissue Among Torpid and Interbout Euthermic Arctic Ground Squirrels. Log normalized transcript abundance measures were pair wise compared among two torpid and two interbout euthermic (IBE) Arctic ground squirrels. Below the diagonal, the gene expression profiles for each pair of individuals are regressed and the traveling local mean is shown in red. The corresponding correlation coefficients for each plot are shown above the diagonal.

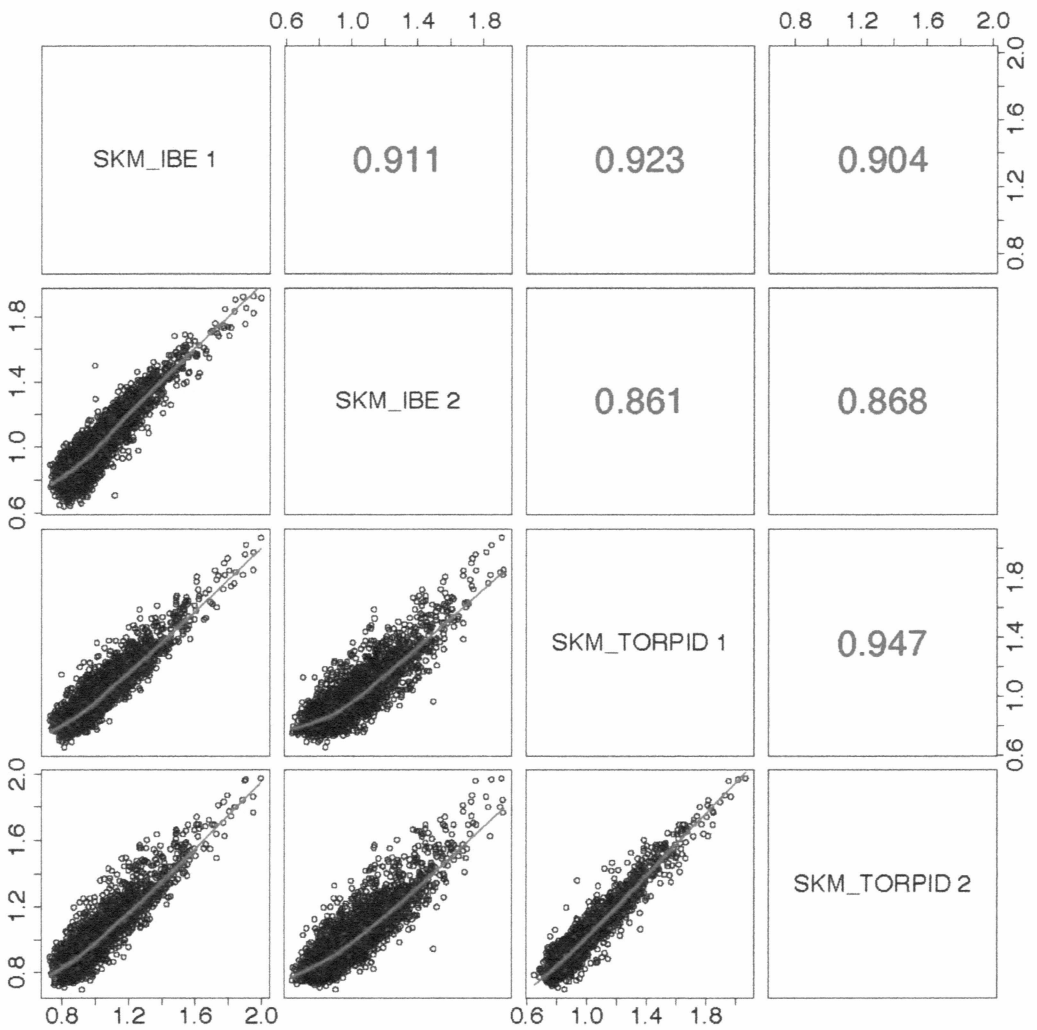

Figure 3.3. Scatter Plot Matrix of Log Normalized Transcript Abundance of Skeletal Muscle Tissue Among Torpid and Interbout Euthermic Arctic Ground Squirrels. Log normalized transcript abundance measures were pair wise compared among two torpid and two interbout euthermic (IBE) Arctic ground squirrels. Below the diagonal, the gene expression profiles for each pair of individuals are regressed and the traveling local mean is shown in red. The corresponding correlation coefficients for each plot are shown above the diagonal.

3.2 Semi-Parametric Analyses of Variance

A majority of the transcripts in both the torpid and the interbout euthermic squirrels appeared near the lower end of the spectrum, showing a general trend toward low variances in transcript abundance in all tissues examined (Figure 3.4). The interbout euthermic squirrels, however, showed a tendency towards larger variance between individuals in transcript expression. The median variance in transcript abundance levels for skeletal muscle, for example, was 2.39×10^{-2} and 2.93×10^{-2} in the torpid and interbout euthermic squirrels, respectively.

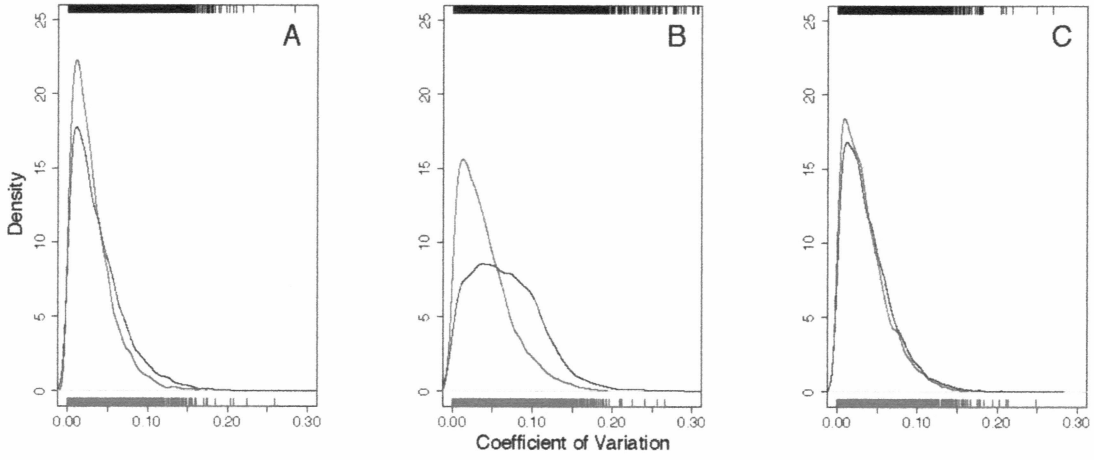

Figure 3.4. Density Distributions of Expression Variation Profiles in Skeletal Muscle, Brown Adipose, and Liver Tissues. The density distributions of the coefficient of variation for gene expression levels are shown in skeletal muscle tissue (A), brown adipose tissue (B), and liver tissue (C). The blue curve outlines the density locations of the torpid Arctic ground squirrels transcripts and the black lines outline those for the interbout euthermic Arctic ground squirrels. Each plot is accompanied by upper and lower ribbons of tick marks indicating the location of each transcript measured at the coefficient of variation level at which it appears along the X-axis.

The variance density plot for brown adipose tissue shows that the transcript levels were still left skewed in samples from both the torpid and interbout euthermic squirrels; less so, however, than in skeletal muscle. Furthermore, the ratio of more highly variant transcripts in interbout euthermic vs. torpid squirrels was also more pronounced. The median variance of the torpid and interbout euthermic was 3.33×10^{-2} and 6.19×10^{-2}, respectively.

The variance density plot for liver tissue was surprisingly similar for torpid and interbout euthermic squirrels, although the tendency for the interbout euthermic liver tissue to have larger variance between individuals in transcript expression compared to torpid squirrels was still present. The median variance of the torpid and interbout euthermic was 2.80×10^{-2} and 3.06×10^{-2}, respectively.

The density distributions of expression variation profiles in skeletal muscle, brown adipose, and liver tissues of torpid and interbout euthermic squirrels showed tissue specificity that may reflect the degree to which these tissues are active during hibernation. Brown adipose tissue, with the largest overall variation in transcript expression levels during torpor and interbout euthermic and the largest difference in variance between torpid and interbout euthermic squirrels, compared to liver and skeletal muscle tissues, is one of the most active tissues during torpor and IBE. The liver tissue is the least active of the three tissues described in this Thesis and has the smallest difference between torpid and interbout euthermic squirrels. The skeletal muscle tissue showed intermediate variance and has an activity level that is between that of brown adipose and liver tissues. These findings are consistent with the correlation matrices as discussed above.

Did higher activity levels of tissues introduce additional random variation simply because of higher temperature in more active tissues? Did active tissues reflect differences among individuals in metabolism that may be related to body size or

slight differences in ambient environmental variables? To answer these questions, future studies could address transcript expression levels measured in torpid and interbout euthermic squirrels that are maintained at different ambient temperatures during hibernation. In addition, elucidating whether squirrels of similar body weights exhibit less variation than those with disparate body weights could assess the effects of body size on transcript expression level variation.

4. Conclusion

This thesis describes a unique way of analyzing high-throughput gene expression screening of two hibernation states. The results are encouraging. They justify a more thorough evaluation of the utility of using global variation in transcript expression patterns. In combination with *a priori* biological knowledge, these patterns will guide future studies into more detailed analyses of hibernation-state dependent and functionally relevant transcripts.

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